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ORIGINAL ARTICLE: NEONATAL LUNG DISEASE

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Postnatal gene expression of airway epithelial sodium transporters associated with birth stress in humans

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Abstract

Introduction: Lung fluid clearance is essential for successful postnatal pulmonary adaptation. The epithelial sodium channel (ENaC) and Na-K-ATPase, induced by serum- and glucocorticoid-inducible kinase 1 (SGK1) as well as aquaporins (AQP), represent key players in the switch from fetal lung fluid secretion to absorption and in early postnatal lung fluid balance. Birth stress, including a surge in catecholamines, promotes pulmonary adaptation, likely through the augmentation of epithelial sodium reabsorption.

Objectives: We sought to determine the changes in the airway gene expression of molecules vital to epithelial sodium transport during early pulmonary adaptation, and the association with birth stress reflected in the norepinephrine concentration in the cord blood in humans.

Methods: We included 70 term newborns: 28 born via vaginal delivery and 42 via elective cesarean section. We determined the norepinephrine concentrations in the cord blood using tandem mass spectrometry and collected nasal epithelial cell samples at 2 min, 1 h, and 24 h postnatally to quantify ENaC, Na-K-ATPase, AQP5, and SGK1 mRNAs using RT-PCR.

Results: The molecular gene expression involved in airway epithelium sodium transport changed markedly within the first hour postnatally. Newborns born via elective cesarean section exhibited a lower expression of ENaC, Na-K-ATPase, and SGK1. Significant correlations existed between the expressions of ENaC, Na-K-ATPase, and SGK1, and the concentration of norepinephrine in the cord blood.

Conclusions: The association of ENaC, Na-K-ATPase, and SGK1 expression with the cord blood norepinephrine concentration points to the importance of birth stress in promoting lung fluid clearance during early postnatal pulmonary adaptation.

KEYWORDS
catecholamines, epithelial sodium channels, ion transport, newborn, norepinephrine, pulmonary adaptation

Abbreviations: AQP, aquaporin; BE, base excess; cAMP, cyclic adenosine monophosphate; CK 18, cytokeratin 18; CS, cesarean section; ENaC, epithelial sodium channel; IS, internal standard; mRNA, messenger RNA; PCR, polymerase chain reaction; VD, vaginal delivery.

1 | INTRODUCTION

Lung epithelial fluid transport undergoes critical changes during the early postnatal period. Fluid movement must reverse from secretion to absorption to enable adequate lung function.[1,2] Infants delivered via elective cesarean section (CS) have a higher content of lung fluid than those delivered vaginally (VD).[3] Impaired lung fluid clearance may result in transient tachypnea of the newborn (TTN), the most prevalent cause of respiratory distress occurring within hours after birth in late preterm and term infants. TTN affects 2.5% of term infants and 10-20% of late preterm infants delivered via CS.[4]

The transition from fetal lung fluid secretion to air breathing represents the integration of different mechanisms, where sodium transport activation in the airway epithelium is a prerequisite of perinatal lung fluid clearance together with the interaction of changing transpulmonary pressure dynamics.[5,6] The first breaths are critical to the clearance of the airspaces immediately following delivery,[7] but fluid reabsorption continues during the initial postnatal hours.[8] In animals, sodium-ion transport is vital in this process.[9,10] The predominant pathway for sodium absorption includes the apical rate-limiting epithelial sodium channel (ENaC) and basolateral Na-K-ATPase, which creates the electrochemical gradient for sodium absorption.[11,12] Sodium gradients result in osmotically driven trans-epithelial fluid absorption, in part via the aquaporin (AQP) water channels.[13]

The switch from lung fluid secretion to absorption during late pregnancy and delivery is timely associated with a surge in glucocorticoid and catecholamine levels.[14–16] Birth stress results in a peak in the catecholamine concentrations.[14–16] Accordingly, among infants delivered via elective CS, the cord blood concentrations of glucocorticoids and catecholamines are lower than those in VD infants.[14–16] In in vitro and in animal studies glucocorticoids and catecholamines or their downstream mediators induce the expression and activity of ENaC, Na-K-ATPase, and AQP5.[17,18] In addition, they activate serum- and glucocorticoid-inducible kinase 1 (SGK1). In animals, increased SGK1 associates with higher expression and activity levels of ENaC and Na-K-ATPase, and enhances lung fluid clearance.[19] However, in humans, data on the molecular mechanisms of lung fluid absorption and their regulation remain scarce.

Therefore, we sought, first, to determine the immediate postnatal expression pattern of airway ENaC, Na-K-ATPase, AQP5, and SGK1 in term newborn humans and, second, to determine whether the gene expression of airway sodium transporters associates with birth stress during the period critical for pulmonary adaptation.

2 | MATERIALS AND METHODS

2.1 | Study population

We included 70 term [median gestational age, 39.4 (38.3-41.9) weeks; Table 1] singleton infants from uncomplicated pregnancies during their first postnatal day; 42 newborns were delivered via elective CS and 28 via VD (Table 1). The main indications for elective CS were a previous CS, a breech presentation, or maternal request.

Infants born to mothers with insulin-dependent diabetes mellitus were excluded. Seven mothers had gestational diabetes, but did not need medication.

The ethics committee of Gynecology and Obstetrics, Pediatrics and Psychiatry at the Helsinki University Hospital approved the study protocol. The parents provided their written consent for inclusion in the study.

2.2 | Sampling and assays

2.2.1 | Cord blood samples

At birth after double-clamping the cord, we obtained arterial and venous cord blood samples in sterile EDTA collection tubes. Samples were kept on ice and plasma was separated by centrifugation at +4°C within 20 min. The plasma samples were stored at −80°C until analysis.

Cord blood arterial ($n = 42$) and venous ($n = 58$) norepinephrine concentrations correlated (Spearman $r = 0.45$, $P = 0.005$). Due to the superior availability of venous samples, further statistical analyses were performed using venous hormone concentrations.

TABLE 1 Clinical characteristics and venous cord blood norepinephrine concentrations

Characteristics	All ($n = 70$)	VD ($n = 28$)	CS ($n = 42$)	P-value
Gestational age (weeks)[a]	39.4 (38.3–41.9)	39.8 (38.3–41.9)	39.3 (38.9–40.4)	<0.001
Birth weight (grams)[a]	3500 (2890–4935)	3667 (2965–4635)	3465 (2890–4935)	0.067
Male sex[b]	42 (60.0%)	15 (53.6%)	27 (64.3%)	0.370
Cord pH[a]	7.29 (7.07–7.44)	7.27 (7.07–7.44)	7.29 (7.18–7.36)	0.075
Cord BE[a]	−0.70 (−9.00–5.00)	−3.6 (−9.00–5.00)	−0.45 (−4.00–2.00)	0.001
Apgar, 1 min	9 (6–10)	9 (6–10)	9 (6–10)	
Norepinephrine (nmol/L)[a]	5.7 (0.8–79.0) $n = 58$	19.0 (2.1–79.0) $n = 23$	4.0 (0.8–19.0) $n = 35$	<0.001

BE, base excess; CS, cesarean section; VD, vaginal delivery.

Data are presented as median (range) or n (%), and groups are compared using the [a]Mann-Whitney U-test, except for gender, for which the [b]chi-square test is used.

P values for comparisons between vaginal delivery and cesarean section.

2.2.2 | Norepinephrine in plasma

We added 20 µL of 100 nM internal standard (IS) (D_6-NA-HCl, D-6634, CDN Isotopes Inc., Pointe-Claire, Canada) to 250 µL of plasma or calibrators containing 0.5-50 nM of norepinephrine. Samples were allowed to stand for 15 min. Calibrators were injected directly into the mass spectrometer.

To a solid-phase microelution plate (Oasis® WCX µElution plate, 30 µm, Waters, Ireland), we added 0.2 mL of methanol and 0.2 mL of water, followed by the samples with IS. The plate was washed with 0.2 mL of 20 mM ammonium acetate followed by 0.2 mL of acetonitrile/isopropanol (50/50). After drying, 2 × 50 µL of 2% formic acid in 95% acetonitrile/water was added, and the eluate was collected.

Next, 35 µL of sample extracts and calibrators were analyzed on a liquid chromatography tandem mass spectrometry system equipped with an API 4000QTrap triple quadrupole mass spectrometer (AB Sciex, Concord, Canada). Peripherals included an Agilent series 1200 high-performance liquid chromatography system with a binary pump (Waldbronn, Germany). Separation was performed on an Atlantis HILIC column (2.1 × 50 mm, 3 µm; Waters, Milford, MA). The mobile phase was 0.2% formic acid in 21% water in acetonitrile, at a flow rate of 300 µL/min. Norepinephrine was detected in the electrospray ionization positive mode with the following transitions: m/z 170.1 to m/z 107.0 and m/z 152.1; and with IS: m/z 176.0 to m/z 158.1. Data were processed using the Analyst Software (version 1.4.2; AB Sciex). The mass calibration and resolution adjustments (at 0.7 atomic mass units at full width and half height) on both resolving quadrupoles were optimized using a polypropylene glycol solution.

Initially, in a subset of patients (n = 5), we measured the levels of both norepinephrine and epinephrine. The values of epinephrine were close to the detection level, rendering them unreliable.

2.2.3 | Nasal epithelial cell samples

We used nasal epithelial scrape samples as a surrogate for distal respiratory epithelium.[20] Airway epithelial cell samples from the nasal epithelium were collected at three time points: 2 (0-6) min, 1.3 (0.8-2.9) h, and 25.5 (24.0-30.4) h [median (range)] postnatally with a sterile probe (RhinoProbe, Arlington Scientific, Springville, UT). Time points were designated as 2 min, 1 h, and 24 h. RNA isolation (RNeasy, Qiagen, Valencia, CA) as well as its spectrophotometric quantitation (NanoDrop, ThermoFisher, Wilmington, DE) were performed as previously described.[21]

We performed the reverse transcription of epithelial cell RNA and PCR of cDNA as previously described.[21] Samples containing genomic DNA were omitted.

Pre-developed TaqMan assays were used for quantitative real-time PCR of α-, β-, γ-ENaC, α1-Na-K-ATPase, AQP5, and SGK1 (Applied Biosystems, Foster City, CA). Cytokeratin 18 (CK18) and β1-Na-K-ATPase primers and probes were designed using the Primer Blast and Beacon designer. Relative amounts of mRNAs were calculated according to the ΔΔCq-algorithm using the epithelial cell–specific CK18 as an endogenous reference gene for normalization.[21]

2.3 | Statistics

Within-group comparisons of the temporal gene expression were performed using Friedman's test with Dunn's post-hoc test. Between-group analyses were performed using the Mann-Whitney U-test, while Spearman's rank-order correlation was used to assess the strength of associations. For figures, linear regression models were fitted for the mRNA variables with the norepinephrine concentration as the explanatory variable. Model fits were assessed visually. Statistical analyses were performed using SPSS Statistics version 23.0 (SPSS, Chicago, IL), and R version 3.5.1.

3 | RESULTS

3.1 | Gene expression of ENaC, Na-K-ATPase, SGK1, and AQP5

Among the entire group of infants, the median expression of α- and β-ENaC, as well as AQP5, decreased between the 2-min to 1-h time points. α-ENaC and AQP5 then increased between 1 and 24 h.

TABLE 2 Relative postnatal mRNA levels of ENaC, Na-K-ATPase, SGK1, and AQP5 for all infants (n = 70), median (IQR)

	2 min	1 h	24 h
α-ENaC	1.66 (1.34-1.97)	1.46 (1.17-1.75)**	1.67 (1.35-2.00)††
β-ENaC	1.45 (1.13-1.77)	1.06 (0.76-1.36)*	0.72 (0.48-0.96)**,†
γ-ENaC	2.41 (1.50-3.32)	1.82 (1.22-2.42)	0.68 (0.39-0.98)**,††
α1-Na-K-ATPase	1.26 (1.07-1.44)	1.09 (0.91-1.28)	0.86 (0.72-0.99)**,††
β1-Na-K-ATPase	1.17 (0.87-1.47)	1.14 (0.82-1.46)	0.98 (0.73-1.24)*,†
SGK1	0.44 (0.22-0.66)	1.04 (0.53-1.54)**	0.74 (0.46-1.03)*,†
AQP5	2.10 (1.43-2.78)	1.47 (0.88-2.06)*	2.27 (1.46-3.07)††

Within-group comparisons are performed using Friedman's test with Dunn's post-hoc test.
*P < 0.05 vs 2 min.
**P < 0.001 vs 2 min.
†P < 0.05 vs 1 h.
††P < 0.001 vs 1 h.

Decreases in γ-ENaC, α1-, and β1-Na-K-ATPase mRNA became statistically significant by the 24-h time point. In contrast, SGK1 expression increased by the 1-h time point and subsequently decreased by the 24-h time point (Table 2).

Significant differences existed between VD and CS infants in the expressions of the ENaC and Na-K-ATPase subunits, as well as in SGK1, but not in AQP5. Compared with VD infants, the expression of γ-ENaC, α1- and β1-Na-K-ATPase, and SGK1 was lower in CS infants at 2 min (all $P < 0.05$). At 1 h, CS infants still had a lower expression of β1-Na-K-ATPase and SGK1, and additionally, the expression of α-ENaC was lower at this time point in the CS group. No differences persisted between the two groups by the 24-h time point (Figure 1).

The expression of ENaC, Na-K-ATPase, SGK1, and AQP5 in infants born to mothers with gestational diabetes ($n = 7$) did not differ from the remaining study population (data not shown). No statistically significant correlations between gene expression and gestational age existed within the present cohort of term infants (data not shown).

3.2 | Associations between birth stress and gene expression

The concentration of norepinephrine in the cord blood was higher after VD than CS ($P < 0.001$; Table 1).

At both the 2-min and 1-h postnatal time points, the expression of the α- and β-ENaC and Na-K-ATPase subunits, as well as the expression of SGK1, associated with the cord blood norepinephrine concentrations (all $P < 0.05$; Figure 2). By contrast, AQP5 expression exhibited no association with norepinephrine.

4 | DISCUSSION

This study documents the early changes in the expression of molecules involved in perinatal sodium absorption in humans. Delivery via CS associated with a lower gene expression of ENaC, Na-K-ATPase, and SGK1 in the airway epithelium during the initial postnatal hours. We also show that the expressions of ENaC, Na-K-ATPase, and SGK1 associate with the cord blood norepinephrine, the predominant catecholamine in fetal circulation.[15] Since during CS without labor stress begins later and the surge in norepinephrine at birth is lower, we argue that the absence of delivery-related stress translates into a molecular-level disadvantage for lung fluid clearance in CS infants during the initial postnatal hours.

The lung fluid content, measured by means of lung ultrasound at 3 h postnatally, is higher in children delivered via CS than via VD.[3] We argue that the lower expression of ENaC, Na-K-ATPase, and SGK1 in CS infants during the first hour postnatally contributes to this finding. Similarly, in line with our previous finding that the difference in lung fluid amounts levels off between the VD and CS infants at 24 h postnatally,[3] no difference in the ENaC, Na-K-ATPase, and SGK1 expressions between CS and VD infants existed at the 24-h time point. The timing of these differences and changes in the gene expression and lung fluid amounts corresponds to that of the emerging symptoms of TTN and their resolution. Compared with healthy infants delivered via VD, infants delivered via CS or those with TTN exhibit a lower ENaC activity in the airway epithelium during the first 24 h postnatally, but no difference persists at 72 h postnatally.[22]

Studies in animals demonstrated the importance of the different roles of ENaC subunits.[9,23,24] The α-ENaC subunit consists of the channel pore and is indispensable for channel function, while the β- and γ-subunits play regulatory roles.[9,25] Indeed, the co-expression of all three subunits augments the ENaC activity manifold.[25,26] Our data show a more pronounced decrease of β- and γ-ENaC expression by the 24-h time point, indicating the significance of regulatory subunits at the moment of birth. Similar to ENaC, the α-Na-K-ATPase subunit forms the pore, while the β-subunit is regulatory.[27] In animals, the gene and protein expression of α1-Na-K-ATPase and β1-Na-K-ATPase peak perinatally[28–30] and the channel activity increases during the last days of gestation, reaching a maximum at term.[29,30] Here, we present novel data on the immediate postnatal expression of Na-K-ATPase subunits in humans. Indeed, they show a higher expression of α1-Na-K-ATPase and β1-Na-K-ATPase within minutes following delivery than at 24 h.

A number of in vitro and animal studies point to the role of catecholamines in perinatal lung fluid clearance. Although disagreement persists regarding the effect of different catecholamines, it is likely that both epinephrine and norepinephrine stimulate lung fluid clearance.[31,32] Beta-adrenergic agonists induce the expression and the activity of ENaC, Na-K-ATPase, and SGK1 in animals and in the laboratory setting.[18,19,33] Furthermore, in lambs, infusing beta-adrenergic agonists during the later stages of lung development induces fluid absorption.[31,34] Likewise, the rise in endogenous catecholamines during labor results in fluid absorption a few hours prior to delivery.[34] cAMP, a second messenger mediating the effects of catecholamines, upregulates α-, β-, γ-ENaC, and α1-Na-K-ATPase mRNAs and proteins.[18,35] Furthermore, reduced amounts of the α1- and β1-NaK-ATPase proteins impair alveolar fluid clearance in response to cAMP.[36]

Based on animal studies, the AQP expression has been associated with the same hormonal factors that implicate lung fluid clearance during the early postnatal adaptation phase.[17,28,37] Interestingly, we found no correlation with AQP5 mRNA and norepinephrine concentrations. Indeed, differences between species in AQP5 expression exist during the perinatal phase. For instance, while in rat lungs, AQP5 expression appeared around birth, in sheep lungs AQP5 expression becomes detectable earlier during the fetal period.[17,28] Recently published data showed increased AQP5 mRNA in gastric aspirates of human infants delivered via CS.[38] However, in relation to delivery-related stress, our data showed no association with nasal airway epithelial AQP5 expression postnatally and the indicator of birth stress during these time points.

Previous findings in humans demonstrated that in preterm newborns with TTN the cord blood concentration of norepinephrine was lower compared with newborns without TTN.[15,39] In this study, we extend this observation to term infants, for whom we demonstrate that the airway epithelial gene expression of ENaC, Na-K-ATPase, and SGK1 associates with the cord norepinephrine concentrations. An

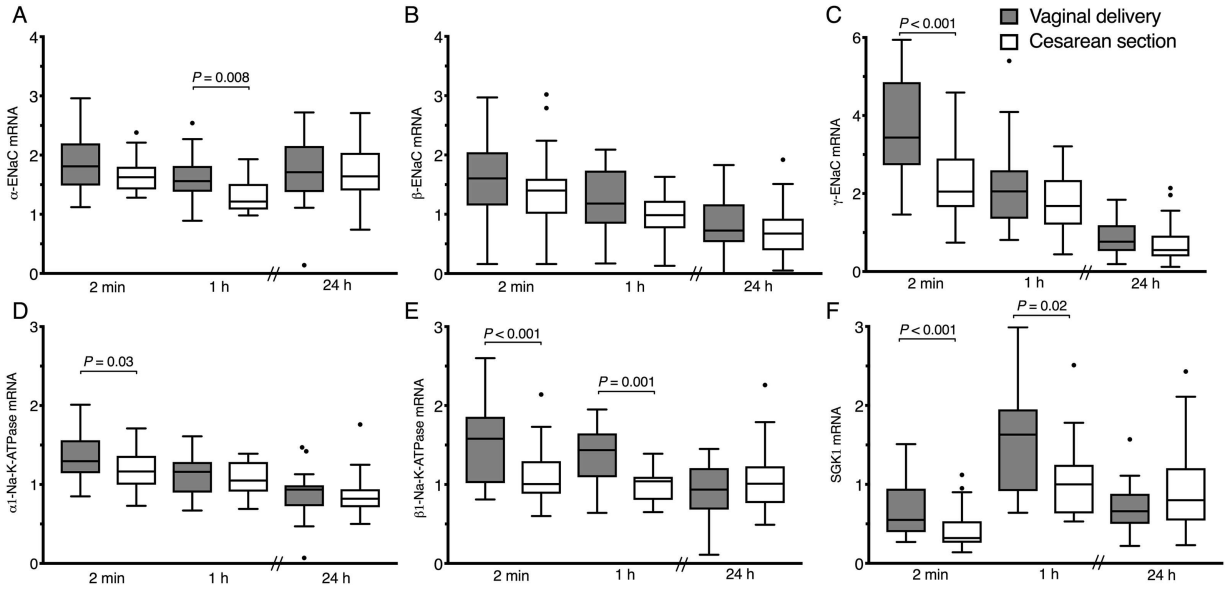

FIGURE 1 Comparison of ENaC, Na-K-ATPase, and SGK1 messenger RNA (mRNA) levels between infants born via vaginal delivery (VD) and cesarean section (CS) at 2 min (VD: $n = 24$, CS: $n = 39$), 1 h (VD: $n = 21$, CS: $n = 19$), and 24 h (VD: $n = 22$, CS: $n = 33$). Between-group comparisons calculated with Mann-Whitney *U*-test and shown with boxplots according to Tukey. (A) α-ENaC, (B) β-ENaC, (C) γ-ENaC, (D) α1-Na-K-ATPase, (E) β1-Na-K-ATPase, and (F) SGK1

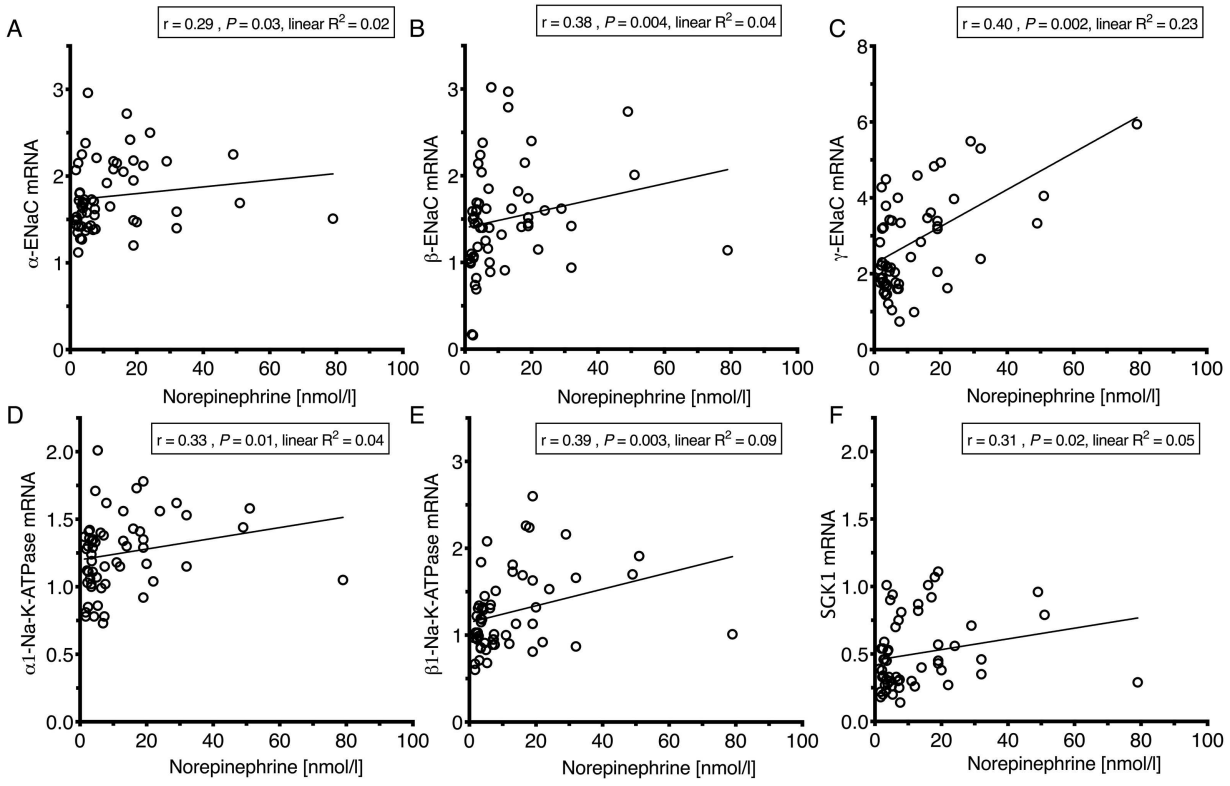

FIGURE 2 Associations of ENaC, Na-K-ATPase, and SGK1 messenger RNA (mRNA) levels with cord blood norepinephrine at 2 min of age ($n = 56$). Correlation coefficient (r) calculated with Spearman's rank order. Linear regression model (R2) fitted for mRNA and norepinephrine and shown as regression line. (A) α-ENaC, (B) β-ENaC, (C) γ-ENaC, (D) α1-Na-K-ATPase, (E) β1-Na-K-ATPase, and (F) SGK1

earlier study demonstrated that early perinatal lung compliance correlates with cord blood catecholamine concentrations.[16] Consistent with this observation, beta-adrenergic agonist administration to mothers prior to delivery improves the lung function of the newborn infant following elective CS.[40] In addition, two clinical intervention studies reported alleviation of respiratory distress following administration of a beta-adrenergic agonist.[41,42]

Our study carries some limitations. First, there is a four-day difference in the gestational age between infants delivered via CS and those delivered vaginally. However, this is unlikely to carry clinical significance. Second, we did not use both norepinephrine and epinephrine levels as an indicator of birth stress. However, norepinephrine has been more closely associated with birth stress than epinephrine.[15,32] Third, we focused on the regulation of and changes in the gene expression. Post-expression regulation of the ion transporters is essential, but its study is primarily limited to in vitro settings. In humans, the gene expression associates with the channel function.[21] Nevertheless, larger studies combining different methods of study of lung fluid clearance during adaptation are warranted.

5 | CONCLUSIONS

In this study, we show that the expression of molecules vital to perinatal lung fluid clearance changes during the early critical phase of pulmonary adaptation in human infants. Different patterns of these changes relate to the mode of delivery. Our study demonstrates that birth stress due to labor is associated with higher cord blood norepinephrine concentrations. In turn, these higher concentrations relate to higher ENaC, Na-K-ATPase, and SGK1 expressions in VD infants.

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CONFLICT OF INTEREST

The authors report no conflicts of interest relevant to this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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